

ROLE OF HEPATITIS C VIRUS INFECTION IN MALIGNANT LYMPHOMA IN SPAIN

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Hepatitis C virus (HCV) has been implicated in the etiology of malignant lymphomas. We estimated the risk of lymphoma associated with detection of HCV infection. Cases ($n = 529$) were consecutive patients newly diagnosed with a lymphoid malignancy between 1998 and 2002 in 4 centers in Spain. Lymphomas were diagnosed and classified using the WHO Classification. Controls ($n = 600$) were hospitalized patients matched to the cases by 5-year age group, gender and study center. Several medical conditions associated with severe immunosuppression precluded the eligibility of controls. Patients underwent a personal interview and blood sampling. HCV positive subjects were considered those with antibody response to third generation ELISA or detection of HCV RNA with Amplicor 2.0. Cases were systematically tested for HIV antibodies. We used the χ^2 test and unconditional logistic regression to estimate the odds ratio (OR) and 95% confidence interval (95% CI) for lymphoma associated with HCV. HCV infection was detected in 40 cases (7.5%) and 23 (3.8%) control subjects. Six of 16 patients with HIV-related lymphomas and 4 of 8 organ-recipient-related lymphomas were HCV positive. The analysis, excluding HIV-infected subjects and organ recipients, led to a prevalence of HCV of 5.9% among cases and 3.8% among controls. The age-, gender- and center-adjusted OR for all lymphomas was 1.58 (95% CI = 0.89–2.79). Among all lymphoma categories, HCV was associated with an increased risk of low grade B-cell lymphomas not otherwise specified (NOS) (OR = 35.98, 95% CI = 4.70–275.4). A 2-fold excess risk associated to HCV was observed for marginal B-cell lymphomas, diffuse large B-cell lymphoma and lymphoma B NOS but the associations were not statistically significant. HCV infection is associated with an increased risk of a broad spectrum of lymphoid neoplasms among non severely immunocompromised subjects in Spain.
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Key words: hepatitis C; lymphoma; case-control studies

Hepatitis C virus is a well-established risk factor in the etiology of liver cancer and of mixed cryoglobulinemia Type II, a condition that can evolve to malignant lymphoma in 8–10% of affected cases. HCV has also been suggested to play a role in the etiology of malignant lymphoma not related to cryoglobulinemia. Data from case–case comparisons and case–control studies indicate several fold higher prevalence of HCV infection (as indicated by the presence of antibodies or HCV RNA) among B-cell lymphoma patients compared to control populations.^{1–7} A prospective study in Japan identified a 1.9-fold increased risk for non Hodgkin's lymphoma among anti-HCV carriers,⁸ and a French cohort observed 3 cases of Non-Hodgkin's lymphoma with chronic HCV when only 0.428 cases were expected.⁹ In contrast, HCV seroprevalence was zero among 57 non-Hodgkin's lymphoma cases an average of 21 years (range = 0–35) before their diagnosis.¹⁰ Follow-up of hemophilic patients has not identified an increased risk for lymphomas associated with HCV, either with or without human immunodeficiency virus (HIV) co-infection.^{11,12} In a recent

study, prevalence of HCV was unrelated to the incidence of lymphoma among 304,411 adults with AIDS.¹³

The objective of our current study was to estimate the risk of lymphoma associated with detection of HCV antibodies (anti-HCV) and HCV RNA in a case–control study that was designed to evaluate the contributions of infectious agents and environmental exposures to the development of this malignancy.

MATERIAL AND METHODS

Subjects

Study subjects were recruited at 4 centers in Spain served by 3 pathology laboratories (1 in Barcelona, 2 in Tarragona [Tortosa and Reus], 1 in Madrid). Age- and gender-standardized prevalence of anti-HCV in the general population was estimated to be 2.5% at these locations.¹⁴ Cases were defined as all consecutive patients having their initial diagnosis of lymphoid malignancy during the study period 1998–2002. The study period included 12 months for the centers in Madrid and Tarragona and 24 months in the Barcelona center. Verification of new diagnosis of lymphoid malignancies within the hematology and pathology departments was done on a daily basis. The diagnosis of lymphoma was verified by histology and 99% of them were supplemented by immunohistochemistry tests and flow cytometry. Diagnosis of chronic lymphocytic leukemia was based on cytology and flow cytometry.¹⁵ Cases were categorized according to the WHO Classification for Neoplastic Diseases of the Lymphoid Tissues¹⁶ and included all B cell, T cell and NK cell neoplasms as well as Hodgkin's lymphoma. Due to the recognized B cell origin of Hodgkin's lymphoma this histological group was also included in some analyses under the category of B cell lymphomas. Subjects with a diagnosis of uncertain malignant potential such as post-transplant lymphoproliferative disorder or monoclonal gammopathies of undetermined significance were excluded. Controls were synchronically identi-

Grant sponsor: Spanish Ministry of Health; Grant number: 98-0066/04; Grant sponsor: European Commission; Grant number: 99CVF2-013, 2000CVG2-011; Grant sponsor: 5th Frame Quality of Life Program; Contract number: QLK4-CT-2000-00422.

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Received 24 June 2003; Revised 17 September 2003; Accepted 24 September 2003

DOI 10.1002/ijc.11727

Published online 31 March 2004 in Wiley InterScience (www.interscience.wiley.com).

fied with the cases and were hospitalized patients matched to the cases by age (± 5 years), gender and study center. Several medical conditions were excluded if they led to hospitalization at the time of interview. Particularly, controls were excluded if the main reason for the hospitalization at the time of recruitment was cancer, organ transplant or systemic infection. HIV carriers and organ transplant recipients were categorized as immunosuppressed subjects and evaluated separately from all other subjects.

Interviews were conducted to collect data on demographic, medical and family history and environmental exposures. Cases and controls provided a blood sample. Informed consent was obtained from all subjects before enrollment, and the Institutional Review Boards of the participating centers approved the study.

Of 700 eligible cases, 529 (75.6%) were included in our present study. Reasons for exclusion were refusal to participate ($n = 28$), death before the interview ($n = 25$), absence of a blood sample ($n = 113$) and absence of an interview ($n = 5$). Of 655 eligible controls, 600 (91.6%) were included in our study. Reasons for exclusion were refusal to participate ($n = 23$) and absence of a blood sample ($n = 32$).

No differences were observed between included and non-included subjects in terms of gender (53.3% vs. 56.4% for males, $p = 0.39$) and number of school years (average years = 9 vs. 10, $p = 0.1$). Not included subjects were slightly older than included subjects (average age = 62 vs. 59, $p = 0.01$).

A centralized pathology review was organized as part of the International Epilymph Study that included a board of 7 pathologists not involved in the original diagnosis. As part of the pre-established protocol a random sample of 20% of the lymphomas within each histological category and within participating centers was reviewed. In addition, all cases with a diagnosis of not-otherwise-specified (NOS) lymphoma were reviewed. In the few cases for which there was a change from the original diagnosis, the panel review was used for the current analysis. Lymphoma cases were not routinely screened for cryoglobulinemia. Cases were systematically evaluated for the detection of antibodies against HIV and history of graft reception.

Site of lymphoma was categorized as nodal or extranodal according to the site of involvement at first diagnosis. Extranodal sites included affected organs other than lymph nodes, bone marrow, spleen and Waldeyer's ring.

Distribution of the medical conditions of the controls included: 14.7% surgical procedures, 14% ocular diseases, 15.6% diseases of the circulatory system, 12% injury and poisoning, 9.1% diseases of the respiratory system, 8.9% diseases of the urogenital system, 8.2% diseases of the gastrointestinal system, 4.1% diseases of the gynecological system, 3.3% infections, 2.6% skin disorders, 2.4% diseases of the liver, 1.9% behavioral problems, 1.4% diseases of the endocrine system, 0.2% diseases of the hematological system and 1.6% diseases of the cerebral system.

HCV infection

From each study subject a serum sample of 200 μ l was stored at or below -80°C until testing for HCV. All tests were done masked to the case-control status. All serum samples were screened for anti-HCV antibodies with a third generation enzyme-linked immunosorbent assay (ELISA) on the AXSYM system according to the manufacturer's instructions (Abbott Laboratories, Wiesbaden, Germany). The assay detects antibodies to putative structural (core region) and non-structural proteins of the HCV genome. Its sensitivity is estimated to be 98.9% (95% CI = 94–100) in patients with chronic liver disease, with specificity of 97.2% (95% CI = 92–99) in panels of sera.¹⁷ In addition, HCV RNA was measured by the licensed Amplicor HCV version 2.0 (Roche Diagnostic, Basel Switzerland) with a lower limit of detection of 50 IU/l. HCV RNA testing was carried out on all seropositive samples ($n = 62$) and a random sample of anti-HCV negative sera ($n = 463$).

HCV infection was defined as a positive test for either anti-HCV or HCV RNA, meaning an ongoing or resolved HCV infection.

Risk associated with HCV viremia, defined by detection of HCV RNA, also was considered. Borderline serological values were considered negative for HCV infection unless HCV RNA was detected. Genotyping was carried out using the Innogenetics Line Probe (Innogenetics, Zwijndrecht, Belgium). Patients were informed of the HCV results through their treating doctor. HIV infection status was determined by testing sera with a licensed commercial ELISA (Abbot Diagnostics, North Chicago, IL). All positive subjects were confirmed with Western blot.

Statistical analyses

Comparison between categorical variables and HCV infection was done by means of a χ^2 test. Two-sided p -values were considered statistically significant at the 0.05 level. Unconditional logistic regression was used to estimate the OR and 95% CI to measure association between specific variables and the risk of lymphoma. All models were adjusted for age (in quintiles), gender and center of diagnosis. The logistic analysis for lymphoma subgroups was carried out comparing each lymphoma subgroup to all controls adjusting for age, gender and center of diagnosis in all models and ignoring in each model all other lymphoma subgroups. A polytomous regression analysis in which all lymphoma categories were included in the same model was also used. The data were analyzed using SPSS version 10.0 and Stata 7.0.

RESULTS

Anti-HCV antibodies were detected in 40 lymphoma cases (7.5%) and in 22 (3.8%) control subjects. Thirteen of 40 (82.5%) of the cases and 18 of 22 controls (81.8%) were both anti-HCV positive and HCV RNA positive, indicating active HCV infection. HCV RNA was detected in one of the 463 (0.21%) anti-HCV negative controls; this subject was considered to be infected with hepatitis C virus.

Table I shows the distribution of subjects by the study design variables, age, gender and diagnosis center. In addition, Table I shows the distribution of cases and controls by the highest educational level attained, reported lifetime history of blood transfusion and reported history of intravenous illicit drug use. No differences were observed between cases and controls in any of the characteristics described in Table I.

TABLE I—DISTRIBUTION OF STUDY SUBJECTS BY AGE, GENDER, AREA OF RECRUITMENT, EDUCATIONAL LEVEL ATTAINED, AND HISTORY OF BLOOD TRANSFUSION AND INTRAVENOUS DRUG USE

	Controls <i>n</i> (%)	Lymphoma cases <i>n</i> (%)
Total	600 (100)	529 (100)
Age		
<43	129 (21.5)	95 (18.0)
43–56	121 (20.2)	106 (20.0)
57–67	126 (21.0)	102 (19.3)
68–74	115 (19.2)	128 (24.2)
>74	109 (18.2)	98 (18.5)
Gender		
Males	311 (51.8)	290 (54.8)
Females	289 (48.2)	239 (45.2)
Recruitment area		
Barcelona	500 (83.3)	416 (78.6)
Madrid	55 (9.2)	68 (12.8)
Tarragona	45 (7.5)	45 (8.5)
Highest educational level attained		
Primary school	237 (53.1)	209 (46.9)
Secondary school	58 (60.4)	38 (39.6)
Higher school	18 (3.0)	20 (3.8)
University degree	41 (6.8)	44 (8.3)
Other (music, military, religion)	38 (6.3)	33 (6.2)
No degree reached	145 (24.2)	115 (21.7)
Never school	63 (10.5)	70 (13.2)
History of blood transfusion	159 (26.7)	129 (24.6)
History of intravenous drug use	8 (1.3)	7 (1.3)

Among cases, 16 were HIV positive of which 6 (37.5%) were also HCV positive. One control was HIV positive and HCV positive. Eight lymphoma cases were recipients of a kidney ($n = 4$) or liver ($n = 4$) allograft. Three of four liver allograft recipients and 1 of 4 kidney recipients were HCV positive. Table II shows the risk of HCV and lymphomas in different inclusion groups. HCV infection was associated with an increased risk of lymphoma when HIV infected patients (OR = 1.78, 95% CI = 1.02–3.11) or organ recipients were excluded (OR = 1.84, 95% CI = 1.08–3.18). When HIV and organ transplanted patients were excluded, the risk for all lymphomas was 1.58 (95% CI = 0.89–2.79). The control for educational level did not modify the results (OR = 1.62, 95% CI = 0.92–2.86). HIV and organ transplanted patients were excluded in the analyses presented thereafter.

The analysis of HCV infection for the different lymphoma categories is presented in Table III. Odds ratios are presented adjusted for the study design variables. Adjustment for age, gender and center of recruitment slightly modified the OR, indicating minor confounding by these variables. Adjustment for socio-demographic or behavioral characteristics did not modify the results. Within B cell lymphomas, an increased risk of low grade B cell NOS was observed (OR = 35.98, 95% CI = 4.7–275.4) although the estimate was based on few subjects. Further, a 2-fold increased risk associated with HCV was seen for marginal zone B cell lymphomas (OR = 3.07, 95% CI = 0.65–14.4) and diffuse large B cell lymphoma (OR = 2.28, 95% CI = 0.87–6.01). The category of "other" B cell lymphomas included several lymphoma types, none of which showed an increased prevalence of HCV. Similar statistical results were obtained by means of the polytomous regression analysis (data not presented). All but 10 HCV-positive subjects had detectable HCV RNA in serum. The OR associated with HCV viremia was 1.65 (95% CI = 0.9–3.1).

All lymphoma patients HCV positive who were not infected with HIV were infected with HCV genotype 1b ($n = 25$, 100%), whereas 76% of all HCV positive controls (12/16) had genotype 1b, 3 (18.7%) had genotype 1a and one (6.2%) had genotype 4c/4d. Seven of nine HIV infected patients with the double infection with HCV had the genotype 1b whereas 2 of 9 had genotype 1a. There was not enough serum in 2 controls to identify the genotype.

Extranodal lymphomas were diagnosed in 68 of 507 (13.4%) cases. In case–case comparisons, HCV prevalence among lymphomas did not differ between extranodal and nodal lymphomas nor did the prevalence of HCV vary by site of lymphoma (e.g., stomach) or stage of lymphoma at diagnosis.

DISCUSSION

Our analysis, comparing a consecutive series of lymphoma cases to hospital-based controls showed that HCV infection was associated with an overall increased risk of lymphoma. The association was particularly strong for low grade B cell lymphoma NOS although based on small numbers. HCV was also associated to other lymphoma categories. More than a 2-fold increased risk was observed for marginal zone B cell lymphoma, diffuse large B cell and B cell lymphoma NOS. These associations were seen among subjects with no HIV infection or history of organ allograft.

We interpret these results as indicative that HCV may play a role in lymphomagenesis among immunocompetent subjects.

Our results are in agreement with previous reports^{5,7,9,18,19} in identifying a moderate increased risk of a wide range of lymphoma types associated to HCV infection. Geographic heterogeneity in HCV genotypes and in ages at and duration of HCV infection, or perhaps small size and low statistical power may account for the failure of some studies to detect an association between lymphoma and HCV infection.^{10,11,13}

This is a large study that includes a systematic testing for HCV of unselected and well defined study populations with careful histological classification of the cases and state-of-the-art laboratory measurements of HCV infection. Furthermore a group of subjects with similar age, gender, education and geographic location with no lymphoma allowed us to estimate the odds of lymphoma associated to HCV infection. In addition, this is the first case–control study exploring the association between HCV and lymphoma carried out in Spain.

As in all hospital based case–control studies, a selection bias can not be ruled out with certainty in our study. We believe, however, that it is unlikely that the results presented can be explained by a major bias in HCV detection or in the selection of subjects. The study included all consecutive patients diagnosed in a definite time period from 1998–2002. We obtained an interview and a blood sample from 75% of lymphoma cases and more than 90% of selected controls. A high inclusion rate and the similar distribution of cases and controls in terms of age, gender, recruitment area, socio-economic status, blood transfusion and intravenous drug use are relevant strengths of our study. Patients that could not be included were of the same gender and educational levels, although slightly older. It is unlikely that this age difference could have resulted in a biased estimate of the association between HCV and lymphoma as no age variation in the HCV prevalence was detected in the included subjects.

One potential limitation of our study is the assessment of HCV status once the disease has been developed. Date of HCV primary infection was unknown. It is likely that most infections were not recent, however, because 20 of 25 of the anti-HCV positive individuals had a first transfusion more than 1 year before enrollment and all intravenous drug users had been addicted for >10 years before the diagnosis.

HCV seroprevalence among control subjects was relatively high (3.8%) due to the fact that our controls were mainly recruited from Spanish inpatients. Previous history of HCV was not an exclusion criterion, but patients were not eligible as controls if they had hepatitis as main diagnosis, or liver cancer. Nonetheless, it is probable that hospital control subjects are more likely to have been exposed to HCV through contaminated blood products more often than the general population. HCV prevalence was previously estimated as 2.5% in the general adult population of Catalonia, the region including Barcelona.¹⁴ The higher prevalence among our controls implies that risk estimates presented may be slightly conservative.

The mechanisms and role of HCV infection in the etiology of lymphoma still remain to be clarified. Recently, HCV has been

TABLE II—OR AND 95% CI FOR LYMPHOMA ASSOCIATED TO HCV INFECTION BY HIV STATUS OR HISTORY OF TRANSPLANT RECIPIENT

	Controls	Cases	OR (95% CI)
HIV negative	22/599	34/513	1.78 (1.02–3.11)
HIV positive	1/1	6/16	NA
Non-transplant recipients	23/600	36/521	1.84 (1.08–3.18)
Transplant recipients	0/0	4/8	NA
Neither HIV or transplant recipient	22/599	30/505	1.58 (0.89–2.79)
Either HIV or transplant recipient	1/1	10/24	NA

¹NA, odds ratio not computed due to empty cells. OR adjusted for age, gender and center of recruitment. Values are HCV positive/total n .

TABLE III – OR AND 95% CI FOR DIFFERENT LYMPHOMA CATEGORIES ASSOCIATED TO HCV INFECTION

Disease category	HCV positive/total ¹	(%)	OR (95%CI) ²
Controls	22/599	3.8	Ref
All lymphomas	30/505	5.9	1.58 (0.89–2.79)
B-cell	28/467	6.0	1.59 (0.89–2.84)
Diffuse large B cell	6/80	7.5	2.28 (0.87–6.01)
Plasma cell myeloma	2/74	2.7	0.64 (0.14–2.84)
Chronic lymphocytic leukemia	7/114	6.1	1.47 (0.59–3.67)
Follicular lymphoma	1/39	2.6	0.87 (0.11–6.85)
Marginal zone B-cell	2/24	8.3	3.07 (0.65–14.4)
Splenic marginal zone	1/25	4.0	0.82 (0.1–6.52)
Lymphoplasmacytic lymphoma	1/21	4.8	1.37 (0.17–11.03)
Low grade B-cell NOS ³	3/5	60.0	35.98 (4.7–275.4)
Lymphoma B NOS	1/5	20.0	3.90 (0.39–39.04)
Hodgkin's lymphoma	3/55	5.5	1.90 (0.51–7.11)
Other B-cell ⁴	1/25	4.0	1.24 (0.15–10.3)
T-cell	2/38	5.3	1.49 (0.33–6.71)

¹Excluded HIV positive subjects and organ transplant recipients. ²OR adjusted for age, gender and center of recruitment. All 599 controls were used to estimate each of the ORs. ³NOS, not otherwise specified. ⁴Other B-cell lymphomas include 9 Mantle cell lymphoma, 2 hairy cell leukemia, 1 Burkitt lymphoma (which was HCV positive), 3 high grade B-cell Burkitt-like, 8 precursor B-lymphoblastic lymphomas, 1 high grade lymphoma B NOS and 1 lymphoma NOS.

shown to infect B cells *in vivo* and to produce an increased rate of apoptotic B cells.²⁰ Moreover, persistence of HCV infection has been associated with chronic stimulation of B cells leading to polyclonal and monoclonal rheumatoid factor-producing cells.¹⁹ The capsid protein of HCV, E2, has been shown to bind to lymphocytes through CD81,²¹ which is associated with CD21 and CD19 proteins on lymphocytes. The adequate binding of these three proteins can lower B cell activation threshold.²² Sustained antigen stimulation has been implicated with aberrant rearrangement of immunoreceptors, resulting in an increased number of cells with chromosomal translocations such as t(14;18) the most common translocation in lymphomas.^{19,23} B cells bearing this translocation have activation of the *bcl-2* gene, increasing the expression of the anti-apoptotic protein bcl-2.

Additional support for the potential role of HCV in lymphomagenesis derives from the observation that effective HCV treatment has been paralleled by reduction of detectable lymphocytes with the t(14;18) translocation^{23,24} and regression of splenic marginal zone lymphoma has also been described after successful treatment of HCV infection.²⁵ Among HCV patients with no mixed cryoglobulinemia, malignant lymphoma has been associated with several histologies, particularly marginal zone lymphoma (mucosa derived, nodal and splenic), follicular lymphoma, multiple myeloma, and diffuse large B cell lymphoma.^{1,26,27} Extranodal lymphoma sites reported in HCV patients include stomach, liver, salivary gland and skin.^{28–30} We did not find that HCV was associated with follicular lymphoma. In our study, the histologies associated with HCV were low grade B cell lymphoma NOS, marginal zone lymphoma, diffuse large B cell lymphoma and B cell lymphoma NOS partially in concordance with the results of others.^{18,30,31} Contrary to that observed in other series,^{28–30} our cases were all of nodal origin. The general lack of histologic specificity favors the hypothesis that HCV may act indirectly in promoting lymphomagenesis and is consistent with the case-control study published recently in Italy where several categories of B-cell lymphomas showed an increased prevalence of HCV.¹⁸ Unlike the study by Mele *et al.*,¹⁸ all the HCV positive lymphoma subjects in our study that could be typed were genotype 1b whereas the highest risk for lymphoma in the Italian series was detected for genotype 2a/2c. Further data are needed to adequately estimate whether risk of lymphoma varies across different genotypes.

Primary or induced immunosuppression is one of the strongest risk factors for lymphomagenesis.³² Acquired immunosuppression is commonly seen in advanced stages of HIV infection and among organ allograft recipients. Lymphomas originating in HIV-HCV coinfecting patients may have a different pathway than other lymphomas.

HIV coinfection with HCV has been shown to stimulate HCV replication and to induce a rapid progression to cirrhosis.^{33,34} It has been suggested that patients with HCV related cirrhosis who undergo liver transplant could have an increased risk of lymphoma as compared to non-HCV infected patients undergoing liver transplant.³⁵ The observation has not been confirmed by others.³⁶

Our definition of an HCV positive individual included anti-HCV positive subjects without HCV RNA and subjects with detectable HCV RNA, indicating resolved and active HCV infection, respectively. More than 80% of the HCV antibody-positive subjects had detectable HCV RNA, confirming the serology and likely indicating ongoing persistent, rather than primary acute, HCV infection. Subjects with antibodies against HCV and no HCV RNA were too few ($n = 10$) to adequately evaluate whether non-viremic HCV infection would have an elevated lymphoma risk. The risk for lymphoma restricted to viremic HCV infection was significantly increased 2-fold for all subjects and non-significantly increased 1.64-fold among non immunocompromised subjects. A larger study is therefore required to investigate whether the risk of lymphoma is only increased in HCV viremic subjects.

Most HCV infections in the population resulted from injection drug use and transfusions given since World War II but before the discovery of the virus and implementation of screening procedures in the early 1990s. Still, the incidence of HCV infection among Spanish blood donors evaluated in 1999 was 3.7/100,000 person-years and the risk of HCV infection was 1/149,000 units transfused.³⁷ These patterns, together with the association that have been observed,^{2,5,18} suggest that chronic HCV infection is associated with the development of lymphomas and that HCV could account for a small proportion of the increasing incidence of lymphoma that has been observed over the last 30–40 years.

In conclusion, our large case-control study clarifies that in Spain HCV infection is associated with an increased risk of lymphoma among non immunosuppressed subjects. The mechanisms of this association need to be further explored.

ACKNOWLEDGEMENTS

This case-control study was undertaken within the framework of the EPILYMPH international study. We thank N. Becker, PhD, for financial support through the Federal Office for Radiation Protection grant. We thank Dr. P. Hartge for her comments; R. Font and Y. Benavente for data clean up, data management and statistical analysis. Polytomous regression analysis was carried out by J. Chen at the National Cancer Institute, USA.

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